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PRINCIPAL INVESTIGATOR: Andrew G. Fraser, Ph.D.
Doctor Julie Ahringer

CONTRACTING ORGANIZATION: University of Cambridge
Cambridge CB2 1TS England

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Andrew G. Fraser, Ph.D.
Doctor Julie Ahringer

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Cambridge
Cambridge CB2 1TS England

Email: agf10@cam.ac.uk

8. PERFORMING ORGANIZATION REPORT NUMBER

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Contents

A. Reiteration of Objectives 4

B. Summary of Achievements 4

C. Results in detail 6

Yeast two-hybrid with BIR-1

Strategy for genome-wide RNAi screen to identify genes with role in cytokinesis

Construction of a genome-wide RNAi library

Screening the RNAi library to identify genes with a cytokinesis defect

D. Other related projects undertaken 7

E. Publications arising from this work 8

F. Figures 9

A. Reiteration of objectives

The *C. elegans* gene *bir-1* was identified as being a homologue of the Inhibitor of Apoptosis Proteins (IAPs). These are known regulators of apoptosis in both insects and mammals. I showed in my previous lab that rather than playing a role in regulating the well-characterised apoptotic machinery in *C. elegans*, *bir-1* instead plays an essential role in cytokinesis. Furthermore, this role appears to be evolutionarily conserved as a mammalian homologue of *bir-1* could functionally complement *bir-1* in *C. elegans*.

I set out to:

(1) Identify *C. elegans* proteins that physically interact with BIR-1 using the yeast two-hybrid assay. Any interactors that I found would be examined using RNAi to determine whether they also played a role in cytokinesis.

(2) Determine where BIR-1 localises in *C. elegans* embryos.

(3) Extend any findings made in *C. elegans* into mammalian systems principally using tissue culture cells.

B. Summary of Achievements

I carried out a yeast two-hybrid screen using both full-length BIR-1 and uniquely the BIR domain as baits. Neither gave any reproducible interactors despite having screened a cDNA library to a reasonable level of saturation (1.5 million and 3 million clones were screened for each bait respectively). The lack of interactors was not due to a lack of expression of my bait: Western blots confirmed that both baits were expressed. The library itself did not appear to be of low quality: other people in the same lab had used the same batch successfully to identify interactors of other proteins. Thus, while there seemed to be no evident defects in the screen, I did not get any genes to work with, and thus could not pursue this avenue any further.

I raised rabbit polyclonal anti-peptide antibodies against BIR-1. These had reasonable ELISA titres and gave detectable signals by Western blot. However, while testing these for immunofluorescence in embryos, a competing group published a paper demonstrating that BIR-1 localised to the chromosomes and spindle midbody. Furthermore, they had evidence that AIR-2, which had an indistinguishable RNAi phenotype to BIR-1, localised to the same regions, and that BIR-1 was required for AIR-2 to localise correctly. There seemed little point in continuing to define the subcellular localisation of BIR-1 myself, since it had already been published, and I therefore decided on a different strategy.

The great similarity in the RNAi phenotypes of *air-2* and *bir-1* suggested that an alternative way to identify other genes that function with *bir-1* was to screen for genes that also gave a cytokinesis defect as an RNAi phenotype. To this end I have:

(1) constructed a library of dsRNA-expressing bacteria that allow us to screen over 85% of all predicted *C. elegans* genes by RNAi just by feeding bacteria to the worms.

(2) Screened this library to identify all genes that, like *bir-1* have embryonic lethal phenotypes.

(3) Participated in a more detailed characterisation of all the embryonic lethal genes. Using 4D time-lapse videomicroscopy, we analyse the RNAi phenotype of all embryonic lethal genes to determine whether they have a cytokinesis defect.

In addition to this, I have conducted an RNAi screen to identify genes that modulate ras signalling and have identified 3 new genes that suppress ras signalling.

These results are all presented in more detail below.

In summary, then, I tried to achieve the immediate goals of my fellowship. However, since these experiments did not pan out as expected, I devised a complementary strategy which is still ongoing. In addition to identifying several genes involved in cytokinesis (and thus candidates for genes that function with *bir-1*), I have used the RNAi library that I built to carry out screens for genes that modulate ras signalling. Ras is a key oncogene in many cancers and thus these screens and their results have direct relevance to the overall research programme of the Breast Cancer Research Programme. This work has resulted in excellent publications (see section F.) including 2 Nature Articles, a Nature paper, a Science paper and a Nature Genetics paper amongst others. I hope that despite deviating slightly from the original objectives due to technical reasons, the committee understand that the best efforts were nonetheless made to get as much scientifically valuable research as possible out of my funding.

C. Results in detail

Yeast two-hybrid with BIR-1

I carried out a yeast two-hybrid screen on a mixed-stage *C. elegans* cDNA library using both full-length BIR-1 and uniquely the BIR domain as baits. Neither gave any reproducible interactors despite having screened the library to a reasonable level of saturation (1.5 million and 3 million clones were screened for each bait respectively). The lack of interactors was not due to a lack of expression of my bait: Western blots confirmed that both baits were expressed. In addition, both baits transactivated reporter genes to some degree, confirming that they also localised to the nucleus as required. The library itself did not appear to be of low quality: other people in the same lab had used the same batch successfully to identify interactors of other proteins. Furthermore, those other screens were carried out at the same time, using the same reagents, so there did not appear to be anything intrinsically defective about the screen. Thus, while there seemed to be no evident defects in the screen, I did not get any genes to work with, and thus could not pursue this avenue any further.

Strategy for genome-wide RNAi screen to identify genes with role in cytokinesis

I showed that embryos in which *bir-1* expression has been greatly reduced using RNAi have a very clear phenotype: they are nonviable and, when observed by timelapse microscopy, have a cytokinesis defect. I reasoned that other genes with a similar RNAi phenotype might play a role in cytokinesis with *bir-1*. To identify such genes, I required first a means to carry out RNAi on all predicted genes in the genome and second a way of analysing them at sufficient throughput to identify all those with a cytokinesis defective phenotype. Since there are over 19,000 predicted *C. elegans* genes, this is a very large task. I focussed on the strategy outlined below.

Construction of a genome-wide RNAi library

It had been shown that when *C. elegans* eat bacteria expressing dsRNA complementary to a gene of interest, this is sufficient to target that gene for RNAi. A library of bacterial strains could thus be constructed in which for each predicted gene there is a single bacterial strain expressing dsRNA against that gene. Thus, by feeding the library of bacterial strains one by one to worms, it is possible to screen the RNAi phenotypes of all predicted genes at high throughput.

We constructed such a library. We established strains for over 85% of predicted genes (16,757 out of 19,213 predicted) using the strategy shown in Fig. 1. In essence, we used PCR to amplify a fragment (size range 300bp-3kb, mean size 1.3kb) from genomic DNA for each gene. These fragments were cloned into the vector L4440, which contains promoters that drive both sense and antisense transcription in bacteria. The resulting construct was transformed into HT115 bacteria, which allow for dsRNA expression and are RNAseIII deficient to ensure that any dsRNA produced is not degraded. A strain was generated for each predicted gene and placed into a specific location in a 96well gridded library. Thus, each gene has a known address within the library. This library can be used for an indefinite number of screens since it consists of bacterial strains, and since to generate an RNAi phenotype using the library only requires feeding worms with bacteria,

several hundred genes can be screened per person per day. This is sufficient throughput to allow a genome-wide RNAi screen.

Screening the RNAi library to identify genes with a cytokinesis defect

Having constructed the RNAi library, we had to devise a way of screening all 16,757 strains individually to determine which genes were required for cytokinesis. Identifying a cytokinesis defective RNAi phenotype is very labour-intensive, requiring careful study and sample preparation. However, all genes with RNAi phenotypes of cytokinesis defects will also be embryonic lethal when targeted. We thus set out to first screen all 16757 genes to identify all those required for embryogenesis — these have embryonic lethal RNAi phenotypes — and subsequently study the detailed phenotype of that subset. In addition to screening for embryonic lethal genes, we also identified genes with other phenotypes, shown schematically in Fig 2. The complete number of genes with each phenotype is given in Table 1.

We identified 937 genes with an embryonic lethal RNAi phenotype — this is probably the great majority of such genes since we detected >70% of previously known embryonic lethal genes. We are in the process of screening these by 4D time-lapse videomicroscopy to identify those with cytokinesis defects. This far, we have screened around half and the list of genes with cytokinesis defects is shown in Table 2. When we have completed this work it will have greatly expanded our knowledge of cytokinesis in *C. elegans* and should provide a list of possible candidates that affect *bir-1*. The genes we identified so far include several genes encoding components of the cytoskeleton including a non-muscle myosin *nmy-2*, a profilin *pfn-1*, *dnc-1* which is part of the dynactin complex, and *tba-2* which is a tubulin. We also identified a subunit of Protein Phosphatase 2A which may play a role in the regulation of cytokinesis and its coordination with spindle assembly. Notably, almost 60% of these have a very similar homologue in another eukaryote (BLAST score better than 1.00*E-10 and length match of at least 80%). This suggests that the genes that we identify in *C. elegans* as having a crucial role in cytokinesis are highly conserved and may play similar roles in other eukaryotes. We are still continuing this work and aim to have completed all analyses by summer 2003. At that point we can begin more directed experiments on the full set of identified genes such as trying to build physical interaction maps, looking at subcellular localisation etc.

D. Other related projects undertaken.

One of the great advantages of the bacterial RNAi library that we generated is that it can be used for many genome-wide screens. We initially screened wild-type animals for phenotypes that are clearly detectable under a relatively low-resolution microscope. However, it is possible to carry out screens for far more subtle phenotypes (including for example the use of GFP reporters): for example, one screen we were involved in used dyes to look at fat metabolism in *C. elegans* — this is not a phenotype that we could have detected in our screen. This screen resulted in the identification of many genes involved in fat metabolism in the worm and was published in Nature.

In addition to screening in a wild-type genetic background, some of the most informative phenotypes are those that are dependent on a mutant background. The ras oncogene is

mutated in a large proportion of human cancers — typically it is locked in an active form. In *C. elegans*, the ras orthologue *let-60* has a mutant allele in which it too is held in an active conformation. This results in ~60% of the adult worms having extra vulvae, the so-called Multivulval (Muv) phenotype. I screened ~1000 genes by RNAi to identify those that in the context of an activated *let-60* allele either caused more worms to become Muv (from 60% to 100%) or that reduced the numbers of Muvs (60% down to 0). Such genes are either candidate suppressors or enhancers of ras signalling. I identified 3 strong suppressors of ras signalling: an orthologue of *S. cerevisiae* SNF2, an orthologue of *S. cerevisiae* YAK1 and a diacylglycerol kinase. All three greatly increase the number of Muv animals in the context of an activated *let-60* allele. Furthermore, I confirmed that the diacylglycerol kinase was a true ras suppressor using a deletion allele. This small pilot screen demonstrated that I can use RNAi screening to identify new genes that modulate ras signalling and I intend to pursue this kind of approach in the coming few years.

E. Publications arising from this work

Fraser AG*, Kamath RS*, Zipperlen P*, Martinez-Campos M, Sohrmann M, Ahringer J.S. Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. Nature 408, 325-30. (2000).

Zipperlen P, Fraser AG, Kamath RS, Martinez-Campos M and Ahringer J. Embryonic roles of *C. elegans* chromosome I genes identified by RNAi. EMBOJ 20(15) 3984-3992. (2001).

Kamath RS, Martinez-Campos M, Zipperlen P, Fraser AG, Ahringer J. Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. Genome Biol 2001;2(1):RESEARCH0002.

Dillin A, Hsu AL, Arantes-Oliveira N, Lehrer-Graiwer J, Hsin H, Fraser AG, Kamath RS, Ahringer J, Kenyon C. (2002) Rates of behavior and aging specified by mitochondrial function during development.Science 298(5602):2398-401.

Kamath RS*, Fraser AG*, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J. (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature 421, 231-237.

Ashrafi K, Chang F, Watts J, Fraser AG, Kamath RS, Ahringer J, Ruvkun G. Genome-wide RNAi analysis of *C. elegans* fat regulatory genes. (2003). Nature, 412, 268-272.

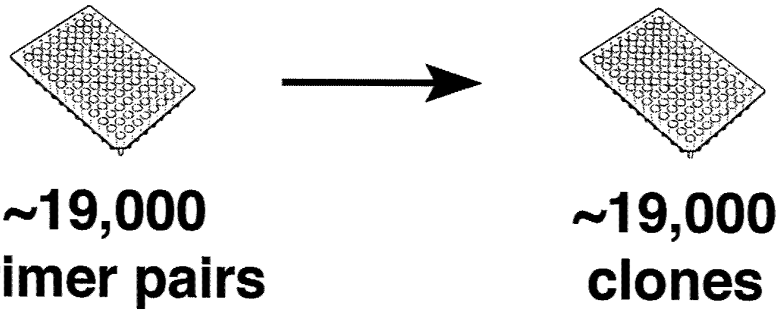

Fig 1a. Goal for bacterial RNAi feeding library
We have ~19,000 oligo primer pairs, one designed to amplify a fragment of each predicted worm gene. We want to make a gridded library of bacterial strains each of which synthesises dsRNA corresponding to the amplified fragment of each gene. These bacterial strains can be fed to worms one by one and we can thus determine the RNAi phenotypes of every gene in the genome.

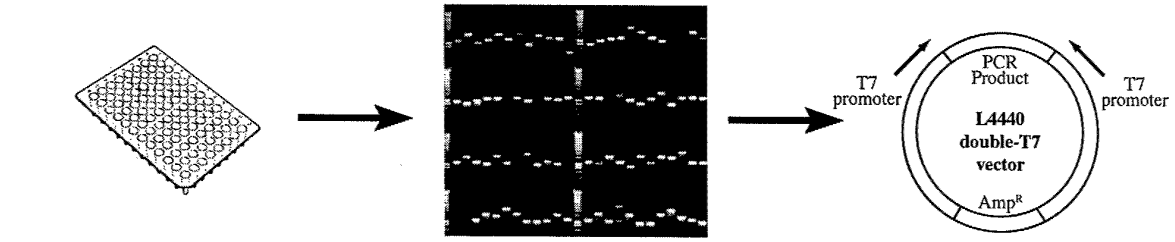

Fig 1b. Outline of cloning strategy
We purchased~19,000 oligo primer pairs, one designed to amplify a fragment of each predicted worm gene from Research Genetics. These were based on sequences designed by Steve Jones. PCR was carried out using Taq on a genomic DNA template — products were this A-tailed and could be cloned using TA-cloning into L4440. Colonies were screened by PCR and glycerol stocks made for all strains.

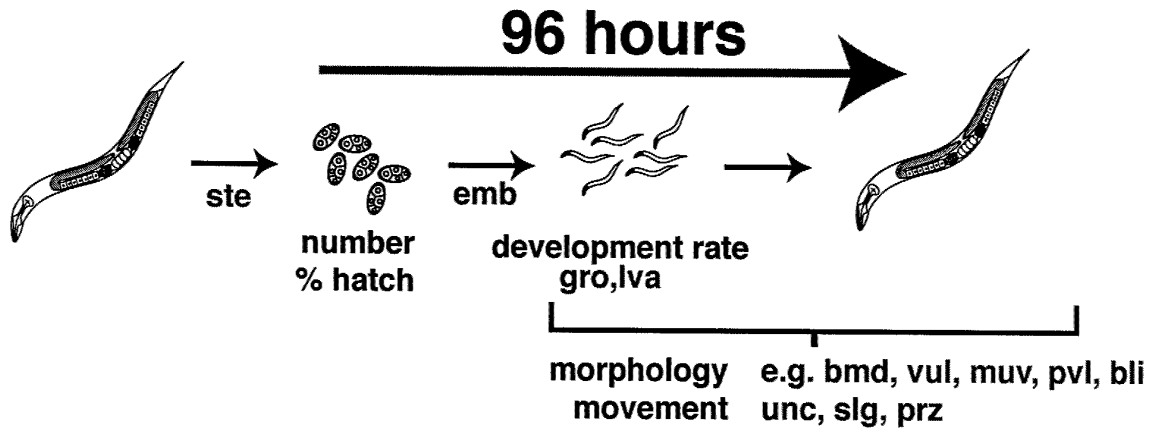

Fig 2. Phenotypes scored in genome-wide RNAi screen
Late larval worms were fed on dsRNA-expressing bacteria. For each gene, the progeny of 3 independent adults were scored at 4 time points over 96hr period (for reference, it takes ~3 days for a fertilised egg to develop to an adult worm) and any genes with phenotypes were repeated again. We scored for Sterility (ste), Embryonic Lethality (emb), delayed development (growth delay 'gro' or larval arrest 'lva') and a variety of post-embryonic defects (these include body morphology defects 'bmd', multivulval 'muv' and blistered cuticle 'bli") as well as movement defects like uncoordinated (unc).

	Chr I (2445 clones)		Chr II (2978 clones)		Chr III (2132 clones)		Chr IV (2693 clones)		Chr V (4152 clones)		Chr X (2357 clones)		TOTAL (16757 clones)	
Phe	Num	%	Num	%	Num	%	Num	%	Num	%	Num	%	Num	%
All	334	13.7	354	11.9	395	18.5	293	10.9	215	5.2	131	5.6	1722	10.3
Nonv	253	10.3	240	8.1	298	14.0	187	6.9	145	3.5	47	2.0	1170	7.0
Grow	37	1.5	41	1.4	59	2.8	70	2.6	46	1.1	23	1.0	276	1.6
Vpep	44	1.8	73	2.5	38	1.8	36	1.3	24	0.6	61	2.6	276	1.6
Emb	226	9.2	204	6.9	220	10.3	143	5.3	108	2.6	28	1.2	929	5.5
Ste	83	3.4	43	1.4	132	6.2	58	2.2	44	1.1	12	0.5	372	2.2
Stp	15	0.6	33	1.1	18	0.8	14	0.5	19	0.5	3	0.1	102	0.6
Gro/Lva	147	6.0	129	4.3	161	7.6	131	4.9	102	2.5	34	1.4	704	4.2
Lvl	38	1.6	61	2.0	35	1.6	18	0.7	20	0.5	24	1.0	196	1.2
Adl	3	0.1	19	0.6	34	1.6	7	0.3	7	0.2	16	0.7	86	0.5
Bli	4	0.2	3	0.1	1	0.0	1	0.0	0	0.0	2	0.1	11	0.1
Bmd	27	1.1	89	3.0	32	1.5	17	0.6	15	0.4	14	0.6	194	1.2
Clr	14	0.6	84	2.8	45	2.1	45	1.7	18	0.4	38	1.6	244	1.5
Dpy	19	0.8	39	1.3	16	0.8	20	0.7	7	0.2	16	0.7	117	0.7
Egl	6	0.2	29	1.0	18	0.8	13	0.5	8	0.2	21	0.9	95	0.6
Him	12	0.5	4	0.1	1	0.0	2	0.1	1	0.0	2	0.1	22	0.1
Lon	2	0.1	11	0.4	9	0.4	8	0.3	3	0.1	5	0.2	38	0.2
Mlt	8	0.3	8	0.3	4	0.2	2	0.1	2	0.0	13	0.6	37	0.2
Muv	2	0.1	4	0.1	5	0.2	2	0.1	0	0.0	1	0.0	14	0.1
Prz	18	0.7	41	1.4	15	0.7	11	0.4	9	0.2	25	1.1	119	0.7
Pvl	32	1.3	39	1.3	37	1.7	17	0.6	13	0.3	9	0.4	147	0.9
Rol	2	0.1	5	0.2	1	0.0	2	0.1	1	0.0	2	0.1	13	0.1
Rup	10	0.4	39	1.3	25	1.2	18	0.7	11	0.3	16	0.7	119	0.7
Sck	6	0.2	32	1.1	67	3.1	44	1.6	25	0.6	6	0.3	180	1.1
Unc	72	2.9	111	3.7	55	2.6	46	1.7	40	1.0	64	2.7	388	2.3

Figure 1 Summary of RNAi phenotypes.
The number of predicted genes with each RNAi phenotype is shown. The Nonv (non-viable; including all phenotypic classes that result in lethality or sterility), Gro (including slow post-embryonic growth or larval arrest), and Vpep (viable post-embryonic phenotype; including all other phenotypic classes) categories are mutually exclusive; however, genes can have multiple specific RNAi phenotypes. Phenotypic classes are Emb (embryonic lethal), Ste (sterile), Stp (sterile progeny), Gro slow post-embryonic growth), Lva (larval arrest), Lvl (larval lethality), Adl (adult lethal), Bli (blistering of cuticle), Bmd (body morphological defects), Clr (clear), Dpy (dumpy), Egl (egg-laying defective), Him (high incidence of males), Lon (long), Mlt (molt defects), Muv (multivulva), Prz (paralyzed), Pvl (protruding vulva), Rol (roller), Rup (ruptured), Sck (sick), and Unc (uncoordinated).described in the Methods. Percentages given are out of the total number of clones screened per chromosome.

Gene	Locus	Description	Function
F26B1.3	ima-2	Member of the karyopherin-alpha protein family	Cell Arch
F20G4.3	nmy-2	Nonmuscle myosin II required for cytokinesis and establishment of early embryonic polarity	Cell Arch
F26E4.8	tba-2	Alpha tubulin specific to meiotic spindles and mitotic spindles of neurons	Cell Arch
Y18D10A.17		Protein with similarity to S. cerevisiae Scd6p, a protein involved in vesicle coat formation	Cell Arch
Y18D10A.20	pfn-1	Member of the profilin protein family	Cell Arch
Y63D3A.5	tfg-1	Protein with strong similarity to C. elegans Y71A12B.F, has similarity to human Hs.180493, TFG protein	Cell Arch
F08D12.1		Protein with strong similarity to C. familiaris SRP72 protein, a signal recognition particle	Cell Arch
Y77E11A.13A		Protein of unknown function	Cell Arch
C33H5.4	klp-10	Putative kinesin-like protein, has strong similarity to human kinesin family member 5B	Cell Arch
ZK593.5	dnc-1	Component of dynactin complex	Cell Arch
ZC518.2		Member of the COPII coat protein, Sec24p-like protein family	Cell Arch
F55G1.10		Member of the histone H2A protein family	Chromatin
R09B3.4		Putative NED-8 conjugating enzyme	Degradation
C47B2.4	pbs-2	Member of the proteasome subunit protein family	Degradation
W08F4.8		Protein with strong similarity to human p50Cdc37 protein	DNA-Cell Cycle
C09G4.3	dom-6	Member of the highly conserved cyclin-dependent kinase regulatory subunit protein family	DNA-Cell Cycle
T23G11.2		Member of the phosphoglucosamine acetyltransferase protein family	Metabolism
F25B4.6		Putative hydroxymethylglutaryl-CoA synthase	Metabolism
Y49A3A.1		Member of the diacylglycerol ethanolaminephosphotransferase protein family	Metabolism
T06G6.9		Protein with strong similarity to human VBP1, which binds the VHL tumor suppressor protein	Protein Synthesis
F39B2.10		Member of the heat shock/DnaJ protein family	Protein Synthesis
C08F8.1		Protein with weak similarity to the human chaperone protein PFDN1	Protein Synthesis
Y45F10A.2	puf-3	Member of the pumilio repeat protein family	RNA Binding
F26A3.3	ego-1	Member of the PTGS (post-transcriptional gene silencing) RdRP (RNA-directed RNA polymerase) gene family	RNA Synthesis
C55A6.9		Protein with weak similarity to S. cerevisiae Paf1p	RNA Synthesis
F38H4.9		Member of the protein phosphatase protein family, predicted to be part of the PP2A core complex	Signalling
K09H11.3		Member of the PH (pleckstrin homology) domain protein family	Signalling
B0025.2		Putative ortholog of human signalosome subunit 2 (SGN2, Hs.30212)	Transcription Factor
T03F1.9	hcp-4	Protein with weak similarity to H. sapiens ROCK1 (Rho-associated, coiled-coil containing protein kinase 1)	Unknown
F56A3.4	spd-5	Putative coiled-coil protein, has weak similarity to S. cerevisiae Uso1p	Unknown
F32H2.3		Protein of unknown function	Unknown
ZK546.1		Putative coiled-coil protein, has weak similarity to S. cerevisiae Uso1p	Unknown
F59E12.11		Protein of unknown function	Unknown
B0547.1		Putative ortholog of human v-jun avian sarcoma virus 17 oncogene homolog JUN	Unknown
F20D12.1		Piwi-related protein with similarity over the C-terminal region to human and D. melanogaster PIWI proteins	Unknown